

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 185 494 B1

(12)

EUROPEAN PATENT SPECIFICATION

(43) Date of publication of patent specification: 20.05.92 (51) Int. Cl.⁵: **C12Q 1/68**

(21) Application number: **85308910.0**

(22) Date of filing: **06.12.85**

(54) **Detection of specific sequences in nucleic acids.**

(30) Priority: **13.12.84 US 681055**

(43) Date of publication of application:
25.06.86 Bulletin 86/26

(45) Publication of the grant of the patent:
20.05.92 Bulletin 92/21

(84) Designated Contracting States:
BE DE FR GB IT SE

(56) References cited:
EP-A- 0 070 685
EP-A- 0 154 505
WO-A-85/04902

BIOTECHNOLOGY, August 1983, pages 471-478, GB; A. KLAUSNER et al.: "Gene detection technology opens doors for many industries"

(73) Proprietor: **APPLIED BIOSYSTEMS, INC.**
850 Lincoln Centre Drive
Foster City California 94404(US)

(72) Inventor: **Whiteley, Norman M.**
151 Highland Avenue
San Carlos, CA 94070(US)
Inventor: **Hunkapiller, Michael W.**
1333 Pebble Drive
San Carlos California 94070(US)
Inventor: **Glazer, Alexander N.**
135 Cannon Drive
Orinda California 94563(US)

(74) Representative: **West, Alan Harry et al**
R.G.C. Jenkins & Co. 26 Caxton Street
London SW1H 0RJ(GB)

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

Description

This invention relates to the probing of specific sequences of nucleotides in a variety of nucleic acid samples, and more particularly to those which contain a sequence characterized by a difference in a single base pair from a standard sequence.

In recent years it has been found that many human diseases can be traced directly to genetic mutations. Some commonly known examples include cystic fibrosis, muscular dystrophy, Tay-Sachs disease, hemophilias, phenylketonuria and sickle-cell anemia. Of the over 500 recognized genetic diseases, many can be traced to single base pair mutations.

Two important techniques have been developed in the art for directly detecting these single base pair mutations. However, neither of these approaches can be easily automated. An automated technique is desirable since it has the potential to decrease labor time, decrease the level of skill required, and should increase reliability. In the first of these prior art techniques, the presence or absence of the mutation in a subject is detected by analysis of a restriction digest of the subject's DNA using Southern blotting. (E. Southern, "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis," *Journal of Molecular Biology*, 98, (1975), 503). For example, sickle-cell disease results from a mutation that changes a glutamic acid residue, coded by the triplet GAG, for a valine residue, coded by GTG, at position 6 in the β -globin chain of hemoglobin. In the mutation of A to T in the base sequence of the β -globin gene, a restriction site for the enzyme MstII (as well as sites for other restriction enzymes) is eliminated. The sickle hemoglobin mutation can therefore be detected by digesting sickle-cell and normal DNA with MstII and using Southern blotting to distinguish the restriction fragments. Normal DNA will generate an MstII fragment 1.1 kilobases long whereas sickle-cell DNA will generate a fragment 1.3 kilobases long.

The specifics of the Southern blot technique are as follows. First, the sample DNA is cut with a restriction enzyme (in this case MstII), and the resultant fragments are separated, based on their size, typically by agarose gel electrophoresis. The gel is then laid onto a piece of nitrocellulose, and a flow of an appropriate buffer is set up through the gel, perpendicular to the direction of electrophoresis, toward the nitrocellulose filter. The flow causes the DNA fragments to be carried out of the gel onto the filter, where they bind, so that the distribution of the DNA fragments in the gel is replicated on the nitrocellulose. The DNA is then denatured and fixed onto the filter. A radioactively labeled probe, complementary to the sequence under study, is then hybridized to the filter, the probe hybridizing to the specific fragment containing the sequence under study. Autoradiography of the nitrocellulose filter then identifies which fragment or fragments contain the sequence under study, each fragment being identified according to its molecular weight. A variation on this technique is to hybridize and do autoradiography directly in the gel, rather than on a nitrocellulose filter. Also, other restriction enzymes may be used provided one of the resulting fragments contains the mutation site of interest.

This direct Southern blot approach used for sickle-cell disease cannot be used, however, for genetic diseases where the mutation does not alter a restriction site, for example, as in α_1 -antitrypsin deficiency, a genetic disease which subjects the individual to greatly increased risk of developing pulmonary emphysema or infantile liver cirrhosis. There, the mutant gene has a single base change (G \rightarrow A) that leads to an amino acid substitution (GLU \rightarrow LYS) at residue 342, thereby producing a non-functional protein. This substitution does not, however, create or destroy a restriction site for any of the currently known restriction enzymes as in sickle-cell anemia. Hence, a straightforward analysis of restriction fragments to search for an altered restriction site is not possible. A technique has been developed, however, which can be used in this situation. (See "Detection of Sickle-cell β -globin Allele by Hybridization with Synthetic Oligonucleotides," by Brenda J. Conner, et al., *Proc. Natl. Acad. Sci.*, Vol 80, pp. 278-282, (Jan 1983), and "Prenatal Diagnosis of α_1 -Antitrypsin Deficiency by Direct Analysis of the Mutation Site in the Gene," by Vincent J. Kidd, et al., *New England Journal of Medicine*, Vol. 310, No. 10, (March 1984).) This second technique is to synthesize a 19-base long oligonucleotide (hereinafter a 19-mer) that is complementary to the normal α_1 -antitrypsin sequence around the mutation site. The 19-mer is labeled and used as a probe to distinguish normal from mutant genes by raising the stringency of hybridization to a level at which the 19-mer will hybridize stably to the normal gene, to which it is perfectly complementary, but not to the mutant gene, with which it has the single base pair mismatch. (By stringency, it is meant the combination of conditions to which nucleic acids are subject that cause the duplex to dissociate, such as temperature, ionic strength, and concentration of additives, such as formamide. Conditions that are more likely to cause the duplex to dissociate are called "higher" stringency, e.g., higher temperature, lower ionic strength, and higher concentration of formamide.) Similarly, if it is desired to detect the mutant gene, instead of the normal gene, a 19-mer is used which is

complementary to the mutant α_1 -antitrypsin sequence around the mutant site. Hence, by using synthetic probes complementary to the sequence of interest in a Southern blot analysis, and varying the stringency, normal and mutant genes can be distinguished.

Although this latter technique is straightforward, it is not without difficulties, especially if an automated procedure is desired. Generally, both the matched and mismatched probes undergo hybridization to the fragment excised by the restriction enzyme, the matched probe being bound at all 19 bases, and the mismatched probe at at most 18 bases. Hence, the relative difference in binding energy between the two probes is quite small. Thus changes in stringency must be delicate enough to dissociate the mismatched probe without also dissociating the matched probe. This would not be a problem with respect to automating the technique were it not for the considerable retention of 19-mer probes by high-molecular weight DNA, presumably due to DNA sequences in the human genome that are somewhat homologous with the synthetic DNA probes used, although this cannot be stated with certainty. This large excess of somewhat homologous sequences in comparison with the α_1 -antitrypsin gene obscures the experimental results and must be treated as background noise in any automated technique and is presently resolved by subjecting the sample to gel electrophoresis and Southern blotting. (See FIGS. 1A and 1B showing the Southern blots for the α_1 -antitrypsin detection scheme reported by Kidd, et al., above.) In this particular instance, this background did not interfere with the diagnosis since a previously developed restriction map indicated that only the band at 2.4 kilobases was relevant. However, it can be seen that most of the probe actually bound is not in the 2.4 kilobase band. In this instance, the time and labor consuming restriction digest and electrophoresis were carried out to separate the DNA sequence of interest, the 2.4 kilobase fragment, from the bulk of the DNA, thereby essentially eliminating background problems.

In most situations involving genetic disease, such restriction maps will already be available, so that the above technique can be quite generally applicable. However, such a technique is not easily automated, just as the previous technique used for sickle-cell disease is not easily automated. What is needed is a technique for detecting single base pair differences between sequences of nucleotides which does not require the use of restriction enzymes, gel electrophoresis, or time consuming autoradiography, and which is readily amenable to automation.

The invention provides a method for diagnosis of genetic abnormalities or other genetic conditions which can be readily automated. The method takes advantage of the low probability that a particular diagnostic sequence which may be found in a number of irrelevant locations in the genome, will be extended by the same or similar contiguous sequence at these irrelevant locations. By requiring that both the diagnostic and contiguous sequence be present, background noise caused by spurious binding of the diagnostic sequence is eliminated, and the necessity for separating the relevant sequence from the background using such techniques as electrophoresis or chromatography is eliminated.

In one aspect, the invention provides a method of probing a target sequence in a sample of denatured nucleic acid which comprises the steps of treating the denatured nucleic acid with a probe complementary to a diagnostic portion of the target sequence (the diagnostic probe), and with a probe complementary to a nucleotide sequence contiguous with the diagnostic portion (the contiguous probe), under conditions under which the diagnostic probe remains bound substantially only to the sample nucleic acid containing the target sequence, covalently attaching the diagnostic probe to the contiguous probe to yield a target probe which is complementary to the target sequence; and removing the unattached probes. Preferably at least one of the probes is labeled. In this way, the presence or absence of the target sequence can then be tested, for example melting the sample nucleic acid-target probe duplex, eluting the dissociated target probe, and testing for the label.

In another approach, the testing is accomplished without first removing probes not covalently attached, by attaching a hook to the probe that is not labeled, so that the labeled target probe may be recovered by catching the hook.

In both instances, the presence of both the diagnostic probe and the contiguous probe is required for the label to appear in the assay. This eliminates the background which had previously been segregated by the size separations accomplished by the Southern blot technique. Hence, the predominant detriments of the prior art techniques have been eliminated, i.e., no treatment with restriction enzymes is required, no gel electrophoresis is required, and no autoradiography is required.

The above method is directly applicable to detecting genetic diseases, particularly those resulting from single base pair mutations, and may be made more precise by comparative results from tests wherein each of the normal and abnormal sequence is made the target sequence. For example, in this embodiment, two diagnostic probes are synthesized, one for the normal gene and one for the mutated gene, and the above method is carried out for each probe independently. The DNA from individuals homozygous for the normal gene will show a high count of label for the probe specific to the normal gene and a low count for the gene

specific for the mutated gene. Similarly, DNA from individuals homozygous for the mutated gene, will show a high count for the probe specific to the mutated gene and a low count for the normal probe. Heterozygous individuals will show a count for both the normal probe and mutated probe which are equal and intermediate between the high and low counts for homozygous and heterozygous individuals. Use of only one diagnostic probe, preferably specific to the mutated sequence of interest is also possible. Using the general method described earlier, the detection scheme is first calibrated so that the amount of label expected corresponding to homozygous normal, homozygous mutant, and heterozygous individuals is known. Then the method is carried out on the sample DNA, and the amount of label detected is compared with the calibration.

FIG. 1A shows the results of a test for α_1 -antitrypsin deficiency. The test used a Southern blot for DNA samples from cloned α_1 -antitrypsin gene (pAT9.6), the MM (normal-normal) and ZZ (deficient-deficient) homozygote cellular DNA controls, the parents, and the fetus at risk, hybridized to a normal M oligomer probe. The band at 2.4 kb indicates the presence of the normal gene.

FIG. 1B shows the same DNA samples hybridized to a deficient Z oligomer probe. The band at 2.4 kb indicates the presence of the deficient gene.

FIGS. 2A, 2B, and 2C illustrate various stages of the method of the invention.

FIG. 3 shows the results of a melting curve analysis of a 15-mer hybridized to a λ -phage DNA sample and of an 80-mer made up of the same 15-mer ligated to a 65-mer which was hybridized adjacent to the 15-mer on the same substrate.

For the purposes of the subsequent description, the following definitions will be used:

"Target sequence" is a nucleic acid sequence, the presence or absence of which is desired to be detected. In the context of a preferred application of the method of the invention, it is a sequence which forms part of a coding region in a gene associated with a genetic disease, such as sickle-cell anemia. In many such diseases, the presence of the genetic aberration is characterized by small changes in the coding sequence; most frequently, normal individuals have sequences which differ by only one nucleotide from the corresponding sequences present in individuals with the genetic "deficiency." In the method of the invention, either the normal or altered sequence can be used as the target sequence.

"Diagnostic portion" refers to that portion of the target sequence which contains the nucleotide modification, the presence or absence of which is to be detected.

"Contiguous portion" refers to a sequence of DNA which is a continuation of the nucleotide sequence of that portion of the sequence chosen as diagnostic. The continuation can be in either direction.

It will be recognized, based on the disclosure below, that the precise position of the selected diagnostic portion is arbitrary, except that it must contain the nucleotide(s) which differentiate the presence or absence of the target sequence. Thus, the contiguous portion continues the sequence of this arbitrarily chosen diagnostic portion.

"Hybridization" and "binding" in the context of probes and denatured DNA are used interchangeably. Probes which are hybridized or bound to denatured DNA are aggregated to complementary sequences in the polynucleotide. Whether or not a particular probe remains aggregated with the polynucleotide depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe.

"Covalently attaching" refers to forming a covalent chemical bond between two substances.

"Ligating" refers to covalently attaching two polynucleotide sequences to form a single sequence. This is typically performed by treating with a ligase which catalyzes the formation of a phosphodiester bond between the 5' end of one sequence and the 3' end of the other. However, in the context of the invention, the term "ligating" is intended to also encompass other methods of covalently attaching such sequences, e.g., by chemical means, and the terms "covalently attaching" and "ligating" will be used interchangeably.

"Probe" refers to an oligonucleotide designed to be sufficiently complementary to a sequence in a denatured nucleic acid to be probed, in relation to its length, to be bound under selected stringency conditions.

"Diagnostic probe" refers to a probe which is complementary to the diagnostic portion.

"Contiguous probe" refers to a probe which is complementary to the contiguous portion.

"Target probe" refers to a probe which is complementary to the target sequence and which is made by covalently attaching (ligating) the diagnostic probe and the contiguous probe.

"Hook" refers to a modification of a probe that enables the experimenter to rapidly and conveniently isolate probes containing this modification by "catching" the hook. The interaction between hook and catching mechanism can be, for example, covalent bonding or ligand receptor binding of sufficient tightness. Such hooks might include antigens which can be recovered by antibody, specific DNA sequences which can be recovered by complementary nucleic acids, and specific reactive chemical groups which can be recovered by appropriate reactive groups.

"Label" refers to a modification to the probe nucleic acid that enables the experimenter to identify the labeled nucleic acid in the presence of unlabeled nucleic acid. Most commonly, this is the replacement of one or more atoms with radioactive isotopes. However, other labels include covalently attached chromophores, fluorescent moieties, enzymes, antigens, groups with specific reactivity, chemiluminescent moieties, and electrochemically detectable moieties, etc.

In a preferred embodiment of the method, certain preliminary procedures are necessary to prepare the sample nucleic acid and the probes before the assay may be performed.

Sample Preparation

The sample nucleic acid is denatured and usually immobilized, typically by being bound to a solid support such as nitrocellulose filter paper. Techniques for denaturing and binding are well known in the art. (See for example, P. 331, Molecular Cloning, by Maniatis, Fritsch, and Sambrook, Cold Spring Harbor Laboratory, 1982, reproduced as Appendix A herein.)

The non-specific binding sites available in the system are then blocked. In the typical case using nitrocellulose filter paper as the solid support, the nucleic acid-paper is treated so that additional nucleic acid will not become irreversibly bound to the paper. This is accomplished, for example, by incubating the nucleic acid and filter for two hours at 80°C in 40x Denhardt's solution (40x Denhardt's solution is 8 g/l bovine serum albumin, 8 g/l polyvinyl pyrrolidone and 8 g/l Ficoll). Then the 40x Denhardt's is removed.

Probe Preparation

In those cases where the diagnostic probe and the contiguous probe are not already available, they must be synthesized. Apparatus for such synthesis is presently available commercially, such as the Applied Biosystems 380A DNA synthesizer and techniques for synthesis of various nucleic acids are available in the literature.

In one embodiment, the probes are prepared for ligation, e.g., if ligase is to be used, the probe which will have its 5' end adjacent the 3' end of the other probe when hybridized to the sample nucleic acid is phosphorylated in order to later be able to form a phosphodiester bond between the two probes. One of the probes is then labeled. This labeling can be done as part of the phosphorylation process above using radioactive phosphorus, or can be accomplished as a separate operation by covalently attaching chromophores, fluorescent moieties, enzymes, antigens, chemiluminescent moieties, groups with specific binding activity, or electrochemically detectable moieties, etc. (Appendix B provides a detailed description for 5' end labeling with ^{32}P using T_4 polynucleotide kinase.)

As another aspect of the invention, the diagnostic and contiguous probes useful for the invention may be packaged as a test kit. A diagnostic probe and a contiguous probe for detecting a particular target sequence associated with a genetic disease are synthesized and one labelled. One of the probes is also terminated in a manner that permits ligation to the other probe. The two probes can then be packaged with appropriate instructions so that the method may be practiced.

Method of the Invention

Step 1.

Hybridize the probe which will remain bound under the more stringent conditions. (Generally, either probe may be longer, and thus remain bound under more stringent conditions. However, for some sequences of nucleotides, the shorter probe may be the one more strongly bound.) The sample nucleic acid is incubated with this probe under conditions that promote specific binding of the probe to only the complementary sequences in the sample nucleic acid.

Step 2.

Hybridize the probe which will remain bound under the less stringent conditions. Again, the sample nucleic acid is incubated, this time with the other probe under conditions which will promote specific binding to only complementary sequences in the sample nucleic acid. Since probe from Step 1 will remain bound under the less stringent conditions required for this probe, hybridization of this probe to the sample nucleic acid will not materially affect the earlier hybridization.

Substep 1a or 2a.

Remove a substantial portion of the diagnostic probe which is not perfectly bound. (If the probe bound in Step 1 is the diagnostic probe, this is Step 1a and should be performed before Step 2, whereas, if the probe bound in Step 2 is the diagnostic probe, this is Step 2a and should follow Step 2.) This is accomplished by washing the sample nucleic acid at an appropriate stringency to dissociate from the nucleic acid any diagnostic probe which is not perfectly bound (i.e., not bound at all sites in the diagnostic portion) while leaving intact that which is perfectly bound. This procedure relies on the fact that there is an energy difference between probes which are perfectly bound and those which are not. In the situation under study, this difference may be quite small, since it can be the result of a single base pair mismatch in an entire diagnostic portion. Hence, the stringency needs to be carefully controlled during this removal process.

Step 3.

Ligate the two probes. The sample nucleic acid with the two probes bound thereto is treated with ligase or treated chemically to covalently attach the two probes, the 5' phosphate of one probe to the 3' hydroxyl of the other probe, at sites where they are hybridized to the nucleic acid adjacent to each other.

Step 4.

Increase the stringency to remove nearly all of the labeled probe that is not ligated to the other probe in Step 3 (> 99%).

Following Step 4, for practical purposes, all that remains in the system is labeled target probe and any of several techniques can be used to detect it. For example, the label can be detected directly by autoradiography or counting, since the background from the non-specific binding of the labeled probe has been removed. In many situations, however, it is preferable and more easily quantitated to further increase the stringency to remove the target probe and to measure the amount of label coming off as the labeled target probe dissociates from the target sequence, a procedure which is easily automated.

In addition, this elution process provides more accurate results than detecting the bound target probe in situ, since it eliminates further background which can be contributed by irreversible binding of the labeled probe directly to the filter paper in those instances where the sample preparation has not been as effective as desired in blocking non-specific binding sites.

FIGS. 2A through 2C illustrate the principles of the above method at several stages, and particularly with regard to the detection of the diagnostic probe. It is assumed, for purposes of discussion, that the contiguous probe is the one which is more tightly bound, and that diagnostic probe is labeled.

FIG. 2A corresponds to the stage immediately after Steps 1 and 2, but before Step 2a, i.e., immediately following hybridization of a diagnostic probe 13 and a contiguous probe 15. In the Figure, the sample nucleic acid, represented by several denatured strands 11, has diagnostic portions D and a contiguous portion C. Where contiguous portion C and diagnostic portion D are adjacent designates the target sequence. Also included are portions X which correspond to any portion of the sample that is sufficiently similar in sequence to the diagnostic portions that the diagnostic probe will hybridize to that section. Areas N indicate any site in the system that will bind probes non-specifically. For purposes of illustration, it is assumed in FIG. 2A that 80% of the diagnostic sequences present will bind diagnostic probe and that 20% of the X sites bind probe and that all N sites bind probe. If the amount of diagnostic probe were measured at this stage to determine the number of target sequences present, there would be excessive background noise as occurred in the prior art due to the binding from all N sites, from D sites outside of the target sequence, and from a percentage of the X sites.

FIG. 2B illustrates the results of the method immediately after Step 4, where the diagnostic and contiguous probes have been ligated to form target probes 17 and the stringency has been increased to the point where all unligated diagnostic probes are no longer hybridized to the sample nucleic acid. If there are only few N sites present at this stage, the amount of the diagnostic probe can be measured in situ to determine the number of target sequences present.

FIG. 2C shows the stage after elution, i.e., where all the probes are removed, including target probes 17. Here, the amount of diagnostic probe which was originally hybridized to the target sequence can be determined without the background noise contributed by the binding to N sites, to X sites, and to D sites not in the target sequence.

The following example is provided as a specific application of the above technique and should not be construed to limit the scope of the invention. Bacteriophage λ -DNA was chosen as the sample nucleic acid in this example for two reasons. First, the expression of the λ genome is generally regarded as a model for genetically determined differentiation processes occurring in cells of higher organisms. Because of its model nature, λ phage was felt to provide an adequate example for a demonstration of the method of the invention. Second, λ phage has been well studied and is readily available.

Example 1

Sample bacteriophage λ -DNA was obtained from Bethesda Research Laboratories in Gaithersburg, Maryland, Catalogue Number 5250. The target sequence chosen corresponds to the sequence of nucleotides starting at base 145 and ending at base 224. (See "Nucleotide Sequence of *cro*, *c II*, and part of the *O* gene in Phage λ -DNA," E. Schwarz, et al., *Nature*, 272, 30 March 1978.)

The diagnostic portion chosen was a 15-mer corresponding to the sequence of nucleotides starting at base 145 and ending at 159 in the λ -DNA. The diagnostic probe, a 15-mer complementary to the diagnostic sequence, was synthesized on an Applied Biosystems 380A DNA synthesizer, and had the sequence 5'-ATCAGCGTTTATAGT-3'. The contiguous portion chosen was a sequence 65 bases long contiguous with the diagnostic sequence, i.e., beginning at base 160 and ending at base 224. The contiguous probe, a 65-mer complementary to the contiguous portion, was also synthesized on an Applied Biosystems 380A DNA synthesizer, and had the sequence 5'-GTTATTTATGCTGTTGTTTTTTTGTACTCGGAAGGGCTTTACCTCTTCGCATAAACGCTTCC-3'. The 15-mer was phosphorylated with ^{32}P using T_4 polynucleotide kinase, thereby also radioactively labeling the 15-mer. (See Appendix B for the specifics of this kinasing process.)

The sample DNA was denatured and immobilized (as described in Appendix A, except that Schleicher & Schuell BA85 nitrocellulose filter was used cut in 9mm diameter circles), and non-specific binding sites were blocked, as described earlier under Sample Preparation. About 0.3 pmol phage λ -DNA was fixed onto the nitrocellulose filter paper during this sample preparation process.

Step 1.

The 65-mer was hybridized to the λ -DNA by incubating the DNA-paper with 150 μl 2xSSC containing 4 pmol 65-mer for 3 hours at 57°C, and the 65-mer solution was removed. (2xSSC is 0.3M NaCl, 0.03M Na citrate pH 7.0.)

Step 2.

The 15-mer was hybridized to the λ -DNA by incubating the DNA paper with 150 μl 2xSSC containing 4 pmol of the labeled 15-mer for 16 hours at 36°C.

Step 2a.

A substantial portion of the 15-mer not perfectly bound (i.e., not bound at all 15 sites) in Step 2 was removed by washing the DNA-paper in 320ml 2xSSC for 10 minutes at 23°C and then washing the filter again in 2 μl of the buffer 66mM Tris HCl at pH 7.5, 6.6mM MgCl_2 , and 10M dithiothreitol.

Step 3.

The two probes were ligated by incubating the DNA-paper in 150 μl of the buffer used in Step 2a, plus 0.4mM ATP and 2 μl T_4 DNA ligase for 2 hours at 23°C.

Step 4.

The stringency was increased to remove nearly all of the labeled probe that was not ligated in Step 4. This was accomplished by washing the DNA-paper, by passing 200 $\mu\text{l}/\text{min}$ of 2xSSC through the filter at 28°C for 1 hour. Although it was designed for another purpose, an Applied Biosystems 470A Protein Sequencer was used for this operation, since it has a cartridge assembly which can hold a filter and can be programmed to deliver liquids through the filter at a programmable temperature.

To detect the ligated probes (the target probe), the stringency was further increased by increasing the temperature 5 °C/hr and passing 0.1xSSC with 0.5% sodium dodecyl sulfate through the filter. Fractions of 200 µl each were collected every 45 minutes. The radioactivity of each fraction was then determined using a liquid scintillation counter and the number of cumulative counts at the end of each interval was plotted versus temperature.

FIG. 3 shows the results of this procedure. For comparison purposes, this was also done for the case where no ligase was used. As can be seen, the use of the ligase dramatically changed the melting curve relative to the unligated case. Essentially all of the unligated labeled probe was dissociated by the time the temperature reached 35 °C. Whereas, in the ligated case, a substantial portion of the labeled 80-mer remained at 35 °C. Hence, by restricting the measurement of radioactivity to material removed from the filter above 35 °C, essentially only ligated probe will be counted. The fact that the 80-mer was all that was being counted above 35 °C was further verified by SDS polyacrylamide gel electrophoresis.

In the context of the general method and the specific example described above, certain aspects of the protocol should be noted. As a general rule, it is preferred that the diagnostic probe be relatively short in absolute terms in order to be able to detect single base pair mismatches. This selectivity is accomplished by removing the diagnostic probe which is not perfectly bound by washing at stringency, as in Step 1a or Step 2a following the hybridization procedure, depending on the step at which the diagnostic probe is hybridized.

In practice, it is of course not practical to remove 100% of the labeled probe not perfectly bound, since to do so would also remove a substantial percentage of the perfectly bound probe. However, for a 15-mer, it is practical to remove over 70% of the labeled probe not perfectly bound, and preferably over 90%. In the usual application of the method, it is only necessary to remove enough imperfectly bound labeled probe to permit differences between homozygous and heterozygous cells to be unequivocally determined. Any imperfectly bound labeled probe which remains hybridized to the target sequence can become ligated and will contribute background noise in the measurements to be made later. Hence, the amount of imperfectly bound labeled probe it is necessary to remove will depend on how much background can be tolerated in arriving at a statistically valid determination.

In the example presented, which is the preferred mode, the labeled probe and the diagnostic probe are the same. A 15-mer was chosen as the diagnostic probe because it was short enough in length to permit any imperfectly bound probe to be readily separated by changes in stringency. The unlabeled probe, i.e., the contiguous probe, was chosen to be sufficiently long to provide a high specificity and a meaningful change in melting point when ligated, and was sufficiently short to hybridize rapidly and could be readily synthesized.

In the unusual situation where the contiguous probe is labeled instead of the diagnostic probe, the diagnostic probe must still be small enough to be able to have specific binding to different alleles which can be differentiated by changes in stringency. Also, the labeled contiguous probe must be small enough to provide a significant change in melting point when ligated to the diagnostic probe so that unligated labeled contiguous probe can be removed before the ligated probe is eluted, while at the same time it must be long enough that the contiguous probe and diagnostic probe together bind to a unique sequence.

From the above discussion, it should also be apparent that the use of a 15-mer and a 65-mer in the example is merely illustrative, and that many other combinations of probe lengths are available. For example, in some situations it may be desirable to use a very short diagnostic probe, perhaps in the range of four to five base pairs in length to further facilitate differentiation of matched and mismatched diagnostic probes by changes in stringency. As a practical matter, however, it should be noted that for the method to offer advantages over the prior art techniques, the length of the ligated probes together must not only be long enough to define a unique sequence to be detected, but also long enough that non-specific binding to sites not in the sequence of interest will not cause excessive background as occurs in the prior art. With regard to the former, clearly the longer the sequence, the more likely it is to be unique. The current art appears to have settled on a definition of a unique sequence as being about 19 nucleotides in length for mammalian genomic DNA, which corresponds roughly to the maximum length which can be reasonably distinguished with presently available techniques using differences in stringency. Given these considerations, at the present time the preferred length for the diagnostic probe ranges from about four base pairs to about nineteen base pairs. With regard to non-specific binding, a minimum combined ligated (target) probe length is unresolved and may vary from gene to gene. From present experiments, however, it appears that an 80-mer is more than adequate, and that an adequate range of lengths would be from about 20 base pairs to about 80 base pairs. In situations, however, where the diagnostic probe is the labeled probe, there appears to be no detriment other than hybridization time and manufacturing considerations to

the use of much longer contiguous probes, for example, up to many thousand base pairs as might be the case if the contiguous probe is a cloned sequence. Hence, in the preferred mode, the lengths for the combined (target) probes ranges upward from about 19 nucleotides.

Some of this size dependency created by using differences in stringency to remove unligated probe can be eliminated by using an alternative technique to recover the ligated probes. For example, it may be useful to provide one of the probes (the unlabeled probe) with a hook so that the ligated (target) probe can be recovered after Step 4 by catching the hook rather than by increasing the stringency. One such approach which appears particularly promising is to attach biotin to the contiguous probe before hybridizing it to the nucleic acid. Steps 1 through 4 in the above method are followed as before and the ligated probe is recovered using streptavidin to bind the biotin molecules contained in the ligated probe. With this approach, relative size is no longer an issue, except that the diagnostic probe must as always be short enough to distinguish single base pair mismatches, and the contiguous probe and diagnostic probe together must be long enough to bind to a unique site. The specifics of this biotin-avidin recovery process which illustrates the general approach are outlined below in Example 2.

15

Example 2

A sample DNA is prepared and the diagnostic and contiguous probes are obtained as previously described. A biotin moiety is then attached to the 3'OH of the contiguous probe, e.g., by using an Enzo Bio-Probe™ Terminal Labeling Kit. Another approach for attaching biotin which is a variation on the Enzo Bio-Probe™ process entails mixing about 1μg of DNA probe with 0.2M potassium cacodylate buffer pH7.0, 1mM CoCl₂, 0.1mM biotinylated dUTP, and incubating for one hour at 37°C or until an average of one biotin per DNA probe has been incorporated. The contiguous probe is then phosphorylated (not labeled) and the diagnostic probe is labeled either at the 5' OH or internally. Steps 1 through 4 are then carried out as before. The recovery process for the ligated probes is effected by increasing the stringency, e.g., by raising the temperature, and then placing the eluted material in close contact with bound avidin, e.g., by passing the eluted material through a porous substrate covalently coated with streptavidin, or incubating the eluted material in a vessel coated with streptavidin. (Streptavidin is an intracellular product of *Streptomyces Avidinii*. See L. Chalet and F. J. Wolf, *Arch. Biochem. and Biophys.* 106: 1-5.) Typical substrates include nitrocellulose filter paper or small beads (generally 3 to 30 microns in diameter). The labeled ligated probe will be concentrated on the porous substrate if and only if it contains the biotin hook (i.e., if a hooked probe and a labeled probe are ligated). This process is particularly useful in concentrating label, for instance, as is often desired with fluorescent labeling schemes. Note also that careful control of temperature is not required to selectively dissociate unligated probe.

Application of the above methods of the invention to the detection of genetic diseases is relatively straightforward. For example, in the detection of sickle-cell disease, it is necessary to distinguish DNA of homozygous individuals with the normal β -globin gene, ($\beta^A\beta^A$), from that of heterozygous individuals with sickle-cell trait having β^S allele, ($\beta^A\beta^S$), from that of individuals having sickle-cell disease, ($\beta^S\beta^S$). In one approach, two diagnostic probes are synthesized, one specific to the normal β -globin (β^A) and one specific to the sickle-cell allele (β^S). Also, the contiguous probe which will be adjacent to these two first probes when hybridized to the test DNA is synthesized and Steps 1 through 4 above are carried out separately for each diagnostic probe. The DNA from individuals homozygous for the normal β -globin gene will then show a high count for the probe specific to the normal gene β^A , and a low count for the probe specific to the β^S allele. Similarly, the DNA from individuals homozygous for the sickle-cell β -globin gene will show a high count for the probe specific to the β^S allele and a low count for the probe specific to the normal gene β^A . DNA from heterozygous individuals ($\beta^A\beta^S$) will show substantially equal counts from both of the specific diagnostic probes, that count in magnitude being intermediate between the high and low counts discussed above for homozygous individuals.

In another approach, a single diagnostic probe specific to the β^S allele can be used in Steps 1 through 4 above, provided adequate controls are used to insure reproducible results. Here total counts are calibrated to distinguish directly normal genotypes without sickle-cell trait or disease (i.e., $\beta^A\beta^A$) from $\beta^A\beta^S$, and $\beta^S\beta^S$ genotypes. In this approach, although it is preferable to use a probe specific to the β^S allele in order to have a positive indication of sickle-cell trait or disease, it should be understood that it is also possible to use a probe specific to the normal genotype to indicate sickle-cell allele, provided that there are no other genetic diseases which might yield a low or intermediate count when hybridized with the normal probe specific to the sequence in the test DNA having a location where the sickle-cell base pair mutation occurs.

While preferred embodiments of the present invention have been described, it will be apparent to those skilled in the art that many changes and modifications may be made without departing from the invention in its broader aspects. For example, it is clear that the contiguous probe may be either direction along the nucleic acid, or that the contiguous probe may be either longer or shorter than the diagnostic probe, depending on the particular target sequence it is desired to identify. Also, the above techniques are useful in determining if a specific sequence is located next to another specific sequence in a DNA chain, or similarly a series of probes each adjacent the next could be used to demonstrate the proximity of specific

10

15

20

25

30

35

40

45

50

55

sequences or to increase the size of the ligated probes.

APPENDIX A

5

IDENTIFICATION OF cDNA CLONES BY HYBRIDIZATION SELECTION 331

10

Binding DNA to Nitrocellulose¹

15

1. Dissolve DNA in water at a concentration of 500 $\mu\text{g/ml}$.
2. Heat to 100°C for 10 minutes.
3. Chill the sample quickly on ice. Add an equal volume of 1 M NaOH and incubate at room temperature for 20 minutes.
4. Using a sterile scalpel and wearing gloves, cut a sheet of nitrocellulose filter (Millipore HAWP) into 3-mm squares. Place the cut filters on the virgin side of a piece of parafilm.
5. Neutralize the DNA sample by adding 0.5 volumes of a solution of 1 M NaCl, 0.3 M sodium citrate, 0.5 M Tris · Cl (pH 8.0), and 1 M HCl. Mix well and immediately chill the DNA sample in ice.
6. Using an automatic micropipette, spot 5 μl of the DNA solution onto each of the filters. Allow it to absorb and then spot another 5 μl . Repeat the process until each filter has been loaded with approximately 20 μg of DNA.
7. Allow the filters to dry in air for an hour.
8. Place the dried filters into a sterile, 50-ml, screw-capped, conical tube. Wash the filters twice with 50 ml of 6× SSC at room temperature. Redistribute the filters onto a fresh sheet of parafilm.
9. Blot the filters dry with Kimwipes. Allow the filters to dry in air for an hour.
10. Place the dried filters into a sterile, glass test tube fitted with a loose metal cap and bake them for 2 hours at 80°C in a vacuum oven. Store the filters at room temperature under vacuum.

20

25

30

35

40

45

50

¹Parnes et al. (1981).

55

Appendix B

Procedure for 5' End Labeling of Probes (Kinasing)

- 5 1. Lyophilize approximately 20 pmol of the probe.
2. Rinse with 10 μ l ethanol and dry sample again to yield salt-free DNA.
- 10 3. Prepare kinase buffer 10x concentrate:
 - 700 mM Tris·HCl, pH 7.6
 - 100 mM MgCl₂
 - 15 1 mM KCl
 - 50 mM Dithiothreitol
4. Dissolve the dried sample in the following solution:
 - 20 For λ -DNA:
 - 1 μ l kinase buffer 10x concentrate
 - 1 μ l unlabeled ATP, 16.5 μ M (or 16.5 pmol)
 - 25 1 μ l spermidine, 10 mM
 - 1 μ l γ -³²P ATP, specific activity 1000 Ci/mmol, concentration 3.3 pmol/ μ l
 - 30 Add H₂O until total volume is 9 μ l.
 - or For Human DNA:
 - 1 μ l kinase buffer 10x concentrate
 - 1 μ l spermidine, 10 mM
 - 35 6 μ l γ -³²P ATP, specific activity 1000 Ci/mmol, concentration 3.3 pmol/ μ l
- Results (both cases) 20 pmoles DNA, 19.8 pmoles ATP
- 40 5. After the sample is dissolved, add 1 μ l of T₄ kinase (e.g., Amersham No. T2020) containing at least 2 units of activity. (Total volume, 10 μ l)
- 50 6. Incubate solution at 37°C for 30 minutes. Freeze.

55

Claims

1. A method of probing a target nucleotide sequence in a sample containing denatured nucleic acid, which method comprises the steps of
 - 5 treating the denatured nucleic acid with a probe complementary to a diagnostic portion of the target sequence and with a probe complementary to a nucleotide sequence contiguous with the diagnostic portion, under conditions under which the diagnostic probe remains bound substantially only to sample nucleic acid containing the target sequence;
 - 10 covalently attaching the diagnostic probe to the contiguous probe to give a target probe complementary to the target sequence; and
 - testing for the presence or absence of the target probe in the sample.
2. A method according to claim 1 which comprises the steps of
 - 15 treating denatured DNA with a first probe which is complementary to a contiguous portion of the target sequence to give an aggregate of first probe and denatured DNA;
 - treating the aggregate with a second probe which is shorter than the first probe and which is complementary to a diagnostic portion of the target sequence;
 - providing conditions of binding under which the first probe does not bind substantially to the denatured DNA which does not contain the contiguous portion and under which the second probe does
 - 20 not bind substantially to the denatured DNA which does not contain the diagnostic portion;
 - ligating the first probe and the second probe;
 - and
 - removing unligated probe.
- 25 3. A method according to claim 1 which comprises the steps of
 - treating denatured, immobilized nucleic acid sample with a first probe complementary to a contiguous portion of the target sequence under conditions of high stringency;
 - treating the resulting product with a second shorter probe complementary to a diagnostic portion of the target sequence under conditions of low stringency under which the second probe does not bind
 - 30 substantially to the denatured nucleic acid which does not contain the diagnostic portion;
 - ligating the first and second probes; and
 - removing unligated probe.
4. A method according to any one of claims 1 to 3, wherein at least one of the diagnostic and contiguous
- 35 probes is labeled.
5. A method according to claim 4, wherein the label is radioactive.
6. A method according to claim 5, wherein the label is ^{32}P at the 5' terminus of one probe to be ligated to
- 40 the 3' terminus of the other probe.
7. A method according to claim 5 or claim 6, which further comprises the subsequent step of detecting the presence or absence of radioactivity in the sample after removing the unattached probes.
- 45 8. A method according to claim 5 or claim 6, which further comprises the subsequent step of measuring the amount of radioactivity present in the sample after removing the unattached probes.
9. A method according to claim 1, which further includes the subsequent step of removing the target
- 50 probe.
10. A method according to claim 9, wherein the target probe is eluted by increasing the stringency.
11. A method according to claim 10, wherein the target probe is eluted by generating a temperature
- 55 gradient.
12. A method according to claim 1, wherein a hook is attached to one of the diagnostic and contiguous probes and the other of the probes contains a label.

13. A method according to claim 12, wherein the target probe is removed by catching the hook.
14. A method according to claim 13, wherein the hook comprises a biotin moiety.
- 5 15. A method according to claim 14, wherein the hook is caught using streptavidin to bind the biotin moiety.
- 10 16. A method according to claim 1, which comprises the steps of
hybridizing one of the diagnostic and contiguous probes which will remain bound to its complementary portion of the target sequence under conditions of higher stringency;
thereafter hybridizing the other of the diagnostic and contiguous probes which will remain bound to its complementary portion in the target sequence under conditions of lower stringency;
removing a substantial portion of the diagnostic probe which is not perfectly bound;
covalently attaching the probes at sites on the nucleic acid where they are bound adjacent to each other to form a target probe complementary to the target sequence;
15 removing unattached probes; and
testing for the presence of target probe in the sample.
- 20 17. A method according to claim 16, wherein the attaching step comprises covalently attaching the 5' end of the diagnostic probe to the 3' end of the contiguous probe.
18. A method according to claim 16, wherein the attaching step comprises covalently attaching the 5' end of the contiguous probe to the 3' end of the diagnostic probe.
- 25 19. A method according to claim 17 or claim 18, wherein the probes are attached using ligase.
20. A method according to any one of claims 16 to 19, further comprising the subsequent step of measuring the amount of target probe in the sample.
- 30 21. A method according to claim 20, wherein the testing step comprises increasing the stringency to remove target probe.
22. A method according to claim 21, which further comprises the subsequent step of measuring the amount of target probe.
- 35 23. A method according to any one of claims 20 to 22, wherein the target sequence corresponds to a genetic mutation from a known normal sequence and wherein the method further comprises the subsequent step of comparing the amount of target probe with a calibrated standard for nucleic acid known to have the genetic mutation.
- 40 24. A diagnostic kit for detecting a target sequence in a nucleic acid sample, the kit comprising
a probe complementary to a diagnostic portion of the target sequence; and
a probe complementary to a nucleotide sequence contiguous with the diagnostic portion of the target sequence, the probes being capable of being ligated.
- 45 25. A kit according to claim 24, wherein at least one of the probes is labeled.
26. A kit according to claim 25, wherein one of the probes is unlabeled and has a hook attached thereto.
- 50 27. A kit according to claim 26, further comprising means for catching the hook.

Revendications

- 55 1. Procédé de sondage d'une séquence nucléotidique cible dans un échantillon contenant un acide nucléique dénaturé, lequel procédé comprend les étapes consistant à :

- traiter l'acide nucléique dénaturé par une sonde complémentaire d'une partie diagnostique de la séquence cible et par une sonde complémentaire d'une séquence nucléotidique contiguë à la partie diagnostique, dans des conditions dans lesquelles la sonde diagnostique reste liée essentiellement seulement à l'acide nucléique de l'échantillon, contenant la séquence cible ;
 - 5 - fixer de façon covalente la sonde diagnostique à la sonde contiguë, pour donner une sonde cible complémentaire de la séquence cible ; et
 - tester la présence ou l'absence de la sonde cible dans l'échantillon.
2. Procédé selon la revendication 1, qui comprend les étapes consistant à :
- 10 - traiter l'ADN dénaturé par une première sonde qui est complémentaire d'une partie contiguë de la séquence cible, pour donner un agrégat de la première sonde et de l'ADN dénaturé ;
 - traiter l'agrégat par une deuxième sonde qui est plus courte que la première sonde et qui est complémentaire d'une partie diagnostique de la séquence cible ;
 - 15 - mettre en oeuvre des conditions de liaison dans lesquelles la première sonde ne se lie pas de façon substantielle à l'ADN dénaturé qui ne contient pas la partie contiguë et dans lesquelles la deuxième sonde ne se lie pas de façon substantielle à l'ADN dénaturé qui ne contient pas la partie diagnostique ;
 - souder la première sonde et la deuxième sonde ; et
 - éliminer la sonde non soudée.
- 20 3. Procédé selon la revendication 1, qui comprend les étapes consistant à :
- traiter un échantillon d'acide nucléique dénaturé immobilisé, par une première sonde complémentaire d'une partie contiguë de la séquence cible dans des conditions de rigueur élevée ;
 - traiter le produit résultant par une deuxième sonde plus courte, complémentaire d'une partie diagnostique de la séquence cible, dans des conditions de faible rigueur dans lesquelles la
 - 25 deuxième sonde ne se lie pas de façon substantielle à l'acide nucléique dénaturé qui ne contient pas la partie diagnostique ;
 - souder les première et deuxième sondes ; et
 - éliminer la sonde non soudée.
- 30 4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel au moins l'une des sondes diagnostique et contiguë est marquée.
5. Procédé selon la revendication 4, dans lequel le marqueur est radioactif.
- 35 6. Procédé selon la revendication 5, dans lequel le marqueur est ^{32}P à l'extrémité terminale 5' de l'une des sondes devant être soudée à l'extrémité terminale 3' de l'autre sonde.
7. Procédé selon la revendication 5 ou la revendication 6, qui comprend en outre l'étape ultérieure de
- 40 détection de la présence ou de l'absence de radioactivité dans l'échantillon après élimination des sondes non fixées.
8. Procédé selon la revendication 5 ou la revendication 6, qui comprend en outre l'étape ultérieure de
- 45 mesure de la quantité de radioactivité présente dans l'échantillon après élimination des sondes non fixées.
9. Procédé selon la revendication 1, qui comprend en outre l'étape ultérieure d'élimination de la sonde cible.
- 50 10. Procédé selon la revendication 9, dans lequel la sonde cible est éluée par augmentation du caractère rigoureux des conditions.
11. Procédé selon la revendication 10, dans lequel la sonde cible est éluée par production d'un gradient de
- 55 température.
12. Procédé selon la revendication 1, dans lequel un crochet est fixé à l'une des sondes diagnostique et contiguë, et l'autre sonde contient un marqueur.

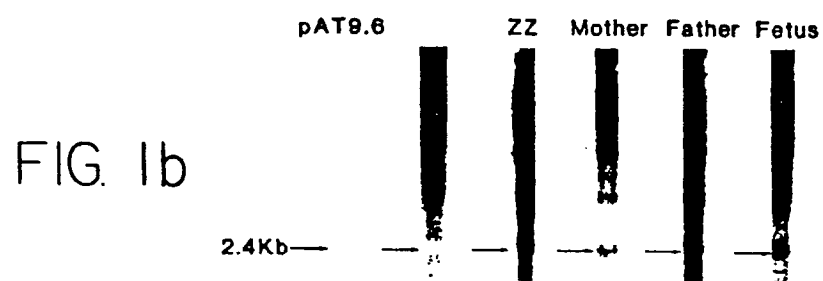
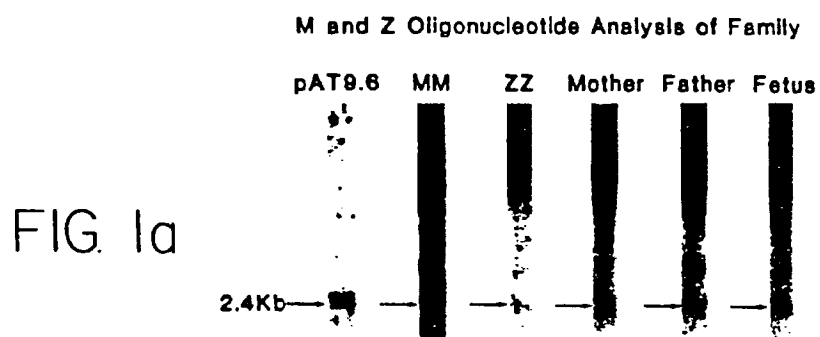
13. Procédé selon la revendication 12, dans lequel la sonde cible est éliminée par saisie du crochet.
14. Procédé selon la revendication 13, dans lequel le crochet comprend une fraction biotine.
- 5 15. Procédé selon la revendication 14, dans lequel le crochet est saisi à l'aide de streptavidine pour lier la fraction biotine.
16. Procédé selon la revendication 1, qui comprend les étapes consistant à :
 - 10 - hybrider l'une des sondes diagnostique et contiguë qui restera liée à sa partie complémentaire de la séquence cible dans des conditions de plus grande rigueur ;
 - hybrider ensuite l'autre des sondes diagnostique et contiguë qui restera liée à sa partie complémentaire dans la séquence cible dans des conditions de rigueur inférieure ;
 - éliminer une partie substantielle de la sonde diagnostique qui n'est pas parfaitement liée ;
 - 15 - fixer de façon covalente les sondes au niveau des sites sur l'acide nucléique où elles sont liées de façon adjacente l'une à l'autre, pour former une sonde cible complémentaire de la séquence cible ;
 - éliminer les sondes non fixées ; et
 - tester la présence de la sonde cible dans l'échantillon.
- 20 17. Procédé selon la revendication 16, dans lequel l'étape de fixation comprend l'opération consistant à fixer de façon covalente l'extrémité 5' de la sonde diagnostique à l'extrémité 3' de la sonde contiguë.
18. Procédé selon la revendication 16, dans lequel l'étape de fixation comprend l'opération consistant à fixer de façon covalente l'extrémité 5' de la sonde contiguë à l'extrémité 3' de la sonde diagnostique.
- 25 19. Procédé selon la revendication 17 ou la revendication 18, dans lequel les sondes sont fixées à l'aide d'une ligase.
20. Procédé selon l'une quelconque des revendications 16 à 19, comprenant en outre l'étape ultérieure de mesure de la quantité de sonde cible dans l'échantillon.
- 30 21. Procédé selon la revendication 20, dans lequel l'étape d'essai comprend l'augmentation du caractère rigoureux des conditions pour éliminer la sonde cible.
- 35 22. Procédé selon la revendication 21, qui comprend en outre l'étape ultérieure de mesure de la quantité de sonde cible.
23. Procédé selon l'une quelconque des revendications 20 à 22, dans lequel la séquence cible correspond à une mutation génétique à partir d'une séquence normale connue et dans lequel le procédé comprend en outre l'étape ultérieure de comparaison de la quantité de sonde cible avec un témoin étalon pour acide nucléique connu pour avoir la mutation génétique.
- 40 24. Coffret pour diagnostique pour détecter une séquence cible dans un échantillon d'acide nucléique, le coffret comprenant :
 - 45 - une sonde complémentaire d'une partie diagnostique de la séquence cible ; et
 - une sonde complémentaire d'une séquence nucléotidique contiguë à la partie diagnostique de la séquence cible,les sondes étant susceptible d'être soudées.
- 50 25. Coffret selon la revendication 24, dans lequel au moins l'une des sondes est marquée.
26. Coffret selon la revendication 25, dans lequel l'une des sondes est non-marquée et porte un crochet.
27. Coffret selon la revendication 26, comprenant en outre des moyens pour saisir le crochet.
- 55

Patentansprüche

1. Verfahren zur Untersuchung einer Zielnukleotidsequenz in einem Prüfling, der denaturierte Nukleinsäure enthält, wobei das Verfahren die folgenden Stufen aufweist:
5 Behandeln der denaturierten Nukleinsäure mit einer zu einem diagnostischen Teil der Zielsequenz komplementären Probe und mit einer zu einer dem diagnostischen Teil benachbarten oder angrenzenden Nukleotidsequenz komplementären Probe, unter Bedingungen, bei welchen die diagnostische Probe im wesentlichen nur mit der Prüflingsnukleinsäure gebunden bleibt, die die Zielsequenz enthält;
10 Kovalentes Binden der diagnostischen Probe mit der angrenzenden Probe, um eine Zielprobe zu erzeugen, die zur Zielsequenz komplementär ist; und
15 Feststellen des Vorhanden- oder Nichtvorhandenseins der Zielprobe in dem Prüfling.
2. Verfahren nach Anspruch 1, aufweisend die folgenden Stufen:
20 Behandeln der denaturierten DNA mit einer ersten Probe, welche zu dem angrenzenden Teil der Zielsequenz komplementär ist, um ein Aggregat der ersten Probe und der denaturierten DNA zu geben;
25 Behandeln des Aggregats mit einer zweiten Probe, welche kürzer als die erste Probe ist und welche zu einem diagnostischen Teil der Zielsequenz komplementär ist;
30 Einstellen von Bedingungen für die Bindung, unter welchen die erste Probe sich im wesentlichen nicht mit der denaturierten DNA bindet, welche den angrenzenden Bereich nicht enthält, und unter welchen die zweite Probe sich im wesentlichen nicht mit der denaturierten DNA bindet, welche den diagnostischen Bereich nicht enthält;
35 Verbinden der ersten mit der zweiten Probe; und
40 Entfernen der ungebundenen Probe.
3. Verfahren nach Anspruch 1, aufweisend die folgenden Stufen:
45 Behandeln des denaturierten, festgelegten Nukleinsäureprüflings mit einer ersten Probe, welche zu dem angrenzenden Bereich der Zielsequenz komplementär ist, unter Bedingungen hoher Stringenz;
50 Behandeln des resultierenden Produkts mit einer zweiten kürzeren Probe, die zu einem diagnostischen Bereich der Zielsequenz komplementär ist, unter Bedingungen niedriger Stringenz, unter welchen die zweite Probe sich im wesentlichen nicht mit der denaturierten Nukleinsäure bindet, welche den diagnostischen Bereich nicht enthält;
55 Binden der ersten mit der zweiten Probe; und
60 Entfernen der ungebundenen Probe.
4. Verfahren nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß wenigstens die eine der beiden Proben, der diagnostischen oder der angrenzenden Probe, markiert ist.
5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß der Markierer radiaktiv ist.
6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß der Markierer ^{32}P am 5'-Ende der einen Probe ist, die mit dem 3'-Ende der anderen Probe zu verbinden ist.
7. Verfahren nach den Ansprüchen 5 oder 6, weiterhin aufweisend die folgende Stufe des Feststellens des Vorhandenseins oder der Abwesenheit von Radioaktivität in dem Prüfling nach dem Entfernen der nicht gebundenen Proben.

8. Verfahren nach den Ansprüchen 5 oder 6, weiterhin aufweisend die nachfolgende Stufe des Messens des Ausmaßes der Radioaktivität, die nach dem Entfernen der nicht gebundenen Proben in dem Prüfling vorhanden ist.
- 5 9. Verfahren nach Anspruch 1, weiterhin aufweisend die nachfolgende Stufe des Entfernen der Zielprobe.
10. Verfahren nach Anspruch 9, dadurch gekennzeichnet, daß die Zielprobe durch Erhöhen der Stringenz gelöst wird.
- 10 11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, daß die Zielprobe durch Erzeugung eines Temperaturgradienten gelöst wird.
12. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß ein Haken an einer der Proben, der diagnostischen oder der angrenzenden Probe, befestigt wird und die andere Probe einen Markierer enthält.
- 15 13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, daß die Zielprobe durch Fangen des Hakens entfernt wird.
- 20 14. Verfahren nach Anspruch 13, dadurch gekennzeichnet, daß der Haken ein Biotinstück enthält.
15. Verfahren nach Anspruch 14, dadurch gekennzeichnet, daß der Haken durch den Einsatz von Streptavidin gefangen wird, um den Biotinteil zu binden.
- 25 16. Verfahren nach Anspruch 1, aufweisend die Verfahrensstufen:
- Hybridisieren einer der Proben, der diagnostischen oder der angrenzenden Probe, welche an ihrem zur Zielsequenz komplementären Teil unter Bedingungen hoher Stringenz gebunden bleibt;
- 30 darauffolgendes Hybridisieren der anderen Probe, der diagnostischen oder der angrenzenden Probe, welche an ihrem zur Zielsequenz komplementären Teil unter Bedingungen niedriger Stringenz gebunden bleibt;
- Entfernen eines wesentlichen Teils der diagnostischen Probe, welche nicht vollständig gebunden ist;
- 35 Kovalentes Binden der Proben an Stellen der Nukleinsäure, wo sie benachbart zueinander gebunden sind, um eine Zielprobe zu bilden, die zur Zielsequenz komplementär ist;
- Entfernen der nicht gebundenen Proben;
- 40 Testen des Vorhandenseins der Zielprobe in dem Prüfling.
17. Verfahren nach Anspruch 16, dadurch gekennzeichnet, daß die Bindungsstufe die kovalente Bindung des 5'-Endes der diagnostischen Probe mit dem 3'-Ende der angrenzenden Probe umfaßt.
- 45 18. Verfahren nach Anspruch 16, dadurch gekennzeichnet, daß die Bindungsstufe die kovalente Bindung des 5'-Endes der angrenzenden Probe mit dem 3'-Ende der diagnostischen Probe umfaßt.
- 50 19. Verfahren nach den Ansprüchen 17 und 18, dadurch gekennzeichnet, daß die Proben durch Einsatz einer Ligase miteinander verbunden werden.
20. Verfahren nach einem der Ansprüche 16 bis 19, weiterhin aufweisend die nachfolgende Stufe des Messens des Anteils der Zielprobe in dem Prüfling.
- 55 21. Verfahren nach Anspruch 20, dadurch gekennzeichnet, daß die Teststufe die Erhöhung der Stringenz umfaßt, um die Zielprobe zu entfernen.

22. Verfahren nach Anspruch 21, aufweisend die nachfolgende Stufe des Messens des Anteils der Zielprobe.
- 5 23. Verfahren nach irgendeinem der Ansprüche 20 bis 22, dadurch gekennzeichnet, daß die Zielsequenz einer genetischen Mutation einer bekannten normalen Sequenz entspricht und daß das Verfahren weiterhin die nachfolgende Stufe des Vergleichs der Menge der Zielprobe mit einem kalibrierten Standard für Nukleinsäure umfaßt, von welcher bekannt ist, daß sie die genetische Mutation enthält.
- 10 24. Diagnostische Ausrüstung zum Feststellen einer Zielsequenz in einem Nukleinsäurepräparat, aufweisend eine zu einem diagnostischen Bereich der Zielsequenz komplementäre Probe; und eine Probe, die zu einer Nukleotidsequenz komplementär ist, die dem diagnostischen Bereich der Zielsequenz benachbart ist, wobei die Proben miteinander verbunden werden können.
- 15 25. Ausrüstung nach Anspruch 24, dadurch gekennzeichnet, daß wenigstens eine der Proben markiert ist.
- 20 26. Ausrüstung nach Anspruch 25, dadurch gekennzeichnet, daß eine der Proben nicht markiert ist und einen an ihr befestigten Haken aufweist.
27. Ausrüstung nach Anspruch 26, gekennzeichnet durch eine Einrichtung zum Fangen des Hakens.



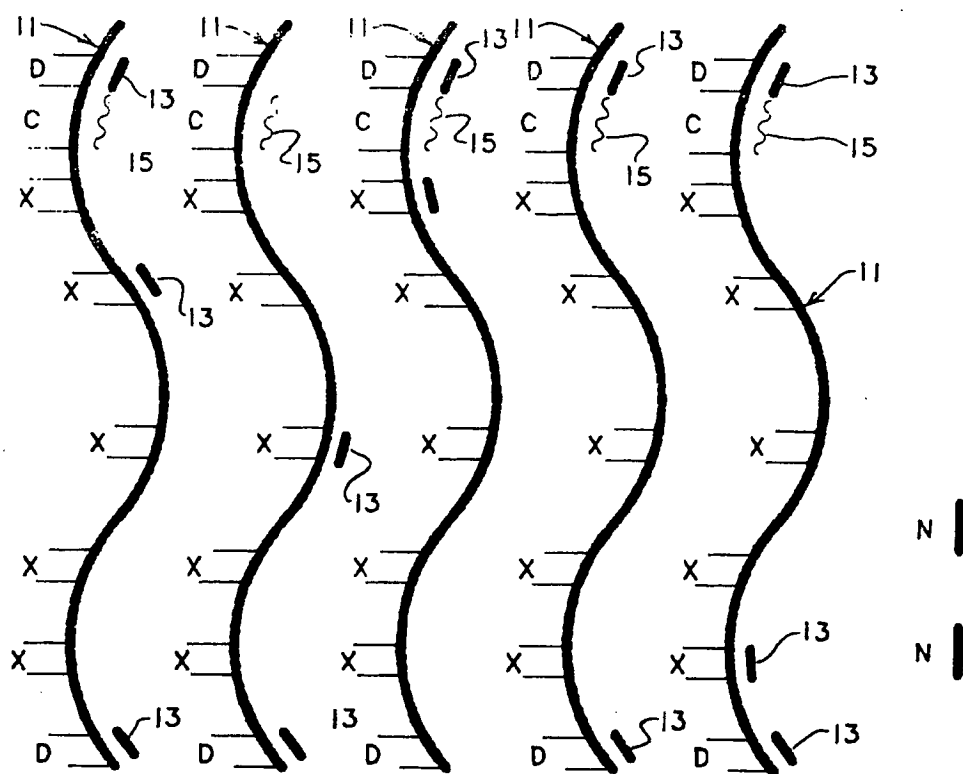


FIG. 2a

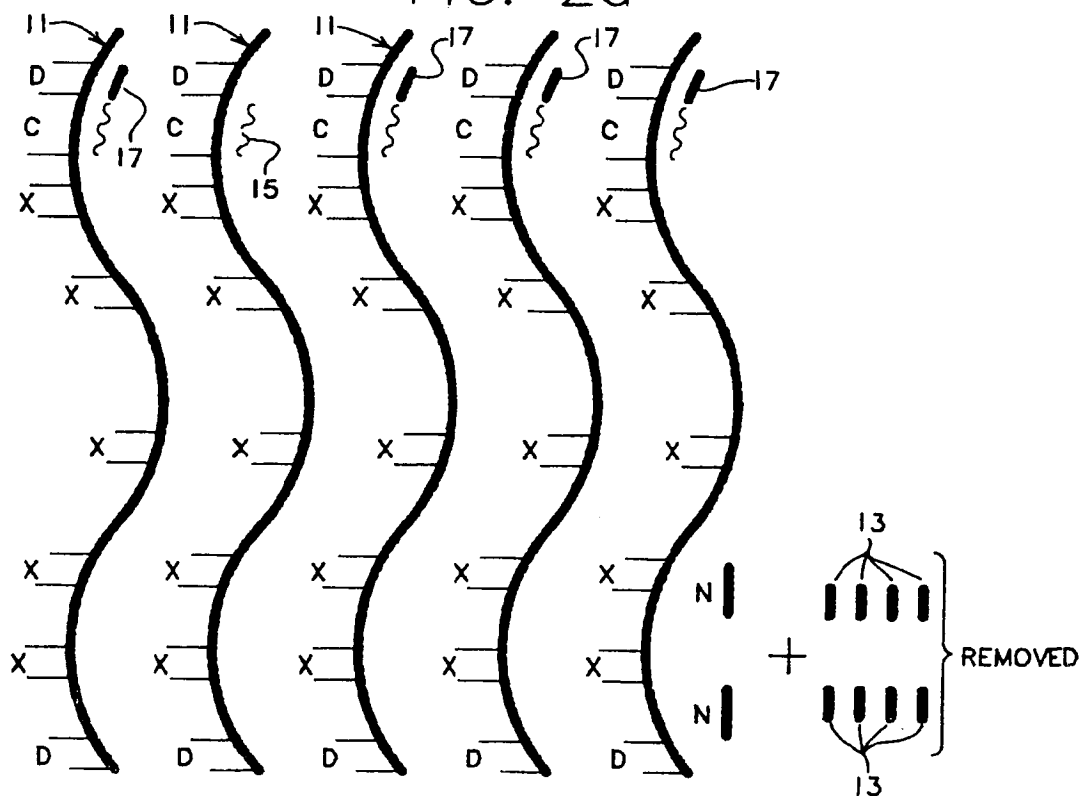


FIG. 2b

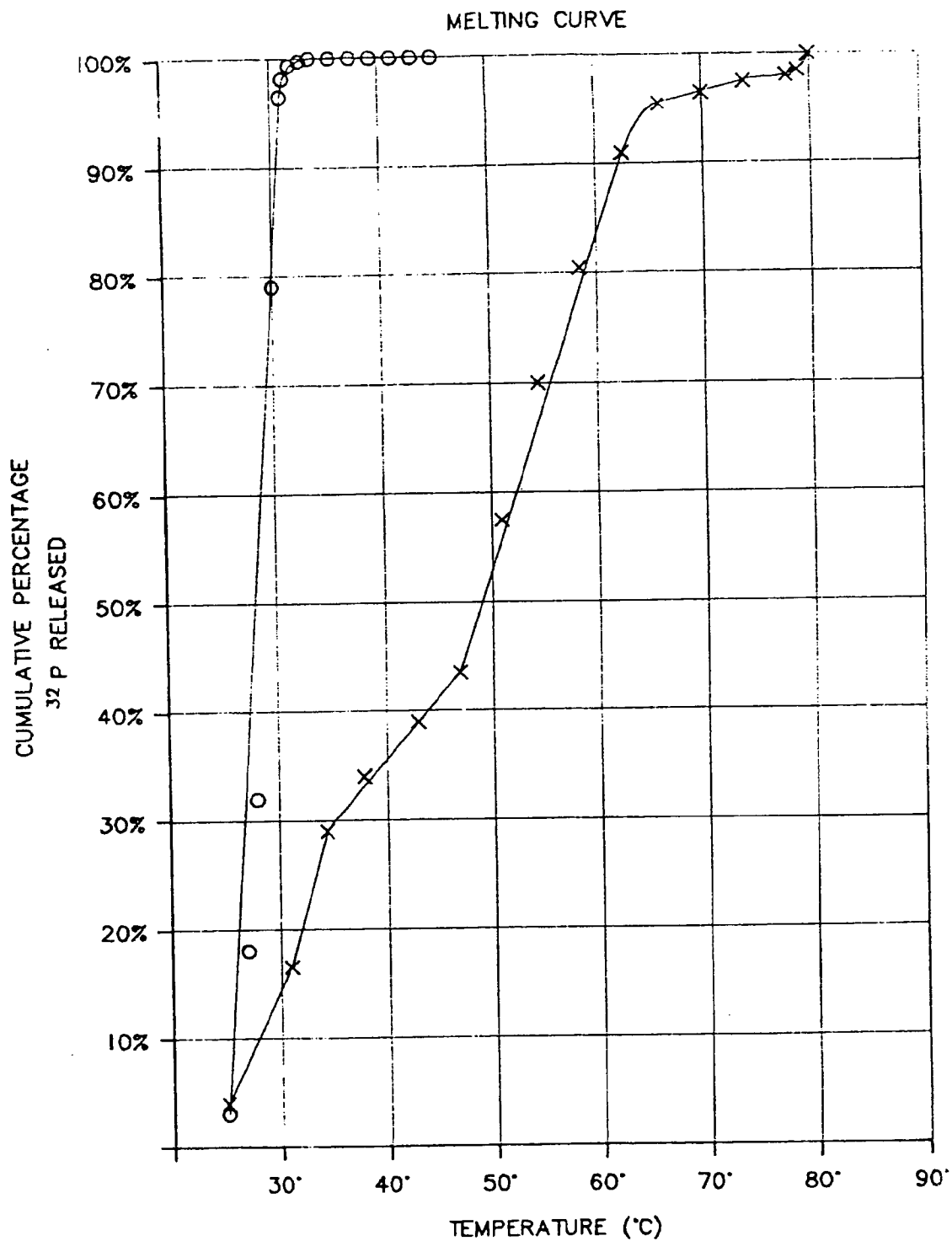


FIG. 3